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13. ABSTRACT ***(Maximum 200 Words)***

Osteoporosis is a disease that afflicts 200 million people worldwide, and that number is expected to increase significantly in the future. Currently, all approved osteoporosis drugs prevent bone loss by interfering with osteoclast function. The greatest therapeutic challenge in the field of osteoporosis is the identification of agents that promote significant bone formation. Fibroblast growth factor 1 (FGF-1) has been shown to be a potent bone anabolic peptide. The goal of this proposal has been to develop mutant forms of FGF-1 that maintain their bone anabolic potential while at the same time reducing its toxic effects (primarily epithelial hyperproliferation) upon systemic administration. Several mutant FGF proteins were developed and their bone anabolic potential compared. Among these Arg 136 » Lys mutant was the most osteoinductive followed by Cys-free > FGF-1 > FGF-HBGAM chimera. Furthermore, we demonstrate that injection of FGF-1 directly into the marrow cavity induces new bone formation suggesting the possibility of local delivery as a strategy to specifically increase the density of bone that are at risk of fracture.

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FOREWORD

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Introduction

This grant was originally awarded to Dr. Wilson Burgess and was transferred to me, due Dr. Burgess' departure from ARC, in June of 2000. The long-term goal of this research program remains to develop fibroblast growth factor-1 (FGF-1) via protein engineering into a potent and specific anabolic agent for the treatment of osteoporosis and fracture repair. Osteoporosis afflicts nearly 200 million people worldwide, and this number is expected to double in the next 20-30 years. It is likely that all people with the disease would benefit from treatments to increase bone mass. The greatest therapeutic challenge in the osteoporosis field at the present time is the identification of agents that promote significant new bone formation. Although there are effective resorption inhibitors for the treatment of osteoporosis (bisphosphanates, estrogens, and calcitonin), these drugs essentially stabilize bone mass but do not lead to substantial increases in bone mass or the restoration of trabecular bone microarchitecture. For patients with severe and established osteoporosis, there is a tremendous need for therapeutic agents that stimulate bone formation and initiate the cascade of events involved in osteoblast differentiation. Those agents that are known to have a stimulatory effect on new bone formation are fluoride, low-dose intermittent parathyroid hormone and its analogs, and the peptide growth factors that are incorporated into the bone matrix and released from bone as it is resorbed.

During the past several years, it has become apparent that members of the FGF family of growth factors and their receptors are essential for normal skeletal growth (1). The preliminary data that formed the basis of the original application demonstrated a significant osteogenic potential for local and systemic administration of FGF-1 *in vivo*. The data also documented certain toxicological or undesirable effects associated with these treatments. Together these data indicated that the therapeutic window is relatively narrow. In the progress report for the first year of funding, Dr. Burgess outlined his progress on the generation of several mutants of FGF-1 and chimeric proteins. He also reported on the *in vitro* and *in vivo* activities of these proteins. Those studies suggested that further evaluation of existing mutants and production of additional mutants or chimeric proteins may improve the efficacy of FGF-1 as an anabolic factor in the treatment of osteoporosis.

The original specific aims of the proposal were:

1) To evaluate the effects of existing mutant forms of FGF-1 on bone cells *in vitro*, on bone formation *in vivo* and to assess their toxicological or undesirable effects.
2) To generate additional FGF-1 mutants or chimeric proteins that are likely to exhibit enhanced anabolic activity on bone with reduced toxicological effects.

In the following, I will summarize the progress that I have made towards these goals since June of 2000.

Body

The crippling effects of osteoporosis primarily manifest themselves in fractures of the femoral neck and vertebral bodies. Based on these clinical observations, we have begun testing the efficacy of various FGF constructs in local bone repair. Our objective is to increase bone mass by injection of a FGF preparation directly into bones that are at the highest risk of fracture. One advantage of this approach is that the required dose of FGF will be substantially less than that required for systemic administration, and therefore the undesirable effects of prolonged high dose systemic administration minimized. Importantly, since the end result would be increased synthesis in critical bones, for example femur, we believe that the clinical benefit will be substantial and potentially equivalent to systemic administration of FGF.

New FGF-1 Constructs: One new FGF-1 construct, Arg 136 » Lys FGF-1, was produced during the current year of support. The rationale behind this construct was as follows. We were interested in testing the bone anabolic efficacy of delivering FGF-1 locally by incorporating the growth factor in fibrin sealant (a mixture of fibrinogen and thrombin produced and marketed by the American Red Cross). We wished to minimize degradation of FGF-1 by thrombin, a protease that is required for clotting of fibrinogen and therefore a necessary component of the fibrin sealant delivery vehicle manufactured by the American Red Cross. Wild type FGF-1 is cleaved by thrombin at Arg 136, and ordinarily we add heparin to protect the FGF from thrombin cleavage. Although heparin renders FGF very resistant to thrombin cleavage, it inhibits mineralization by osteoblast cultures in vitro, and causes osteoporosis in vivo. For this reason, the thrombin cleavage site of FGF-1, Arg 136 was mutated to a Lys. In order to test if Arg 136 »

Lys mutant is more thrombin resistant 20 μg of it or the wild type FGF-1 were incubated with 6 units of thrombin for various times as indicate in Fig.1. The samples were then subjected to SDS-PAGE followed by coomassie blue staining, and the amount of intact vs. degraded FGF in each lane assessed. The data presented in Fig.1 clearly demonstrate that the Arg 136 » Lys mutant is significantly more resistant to thrombin than normal FGF-1.

Next we established that the Arg 136 » Lys FGF-1 maintains its mitogenic activity. In Fig.2, indicated amounts of FGF-1 or Arg136 » Lys FGF-1 were added to NIH3T3 cells for 22 hours. During the last four hours of incubation, 2 μCi of ^{3}H-thymidine was added to each well; subsequently the cells were harvested and the amount of label incorporated into DNA was quantitated. The results illustrate that the Arg 136 » Lys FGF-1 has identical mitogenic activity to the wild type protein.

Localized bone induction. The basic protocol has been to present FGF, or various mutant forms of it in a slow release form juxtaposed to calvarial bones for two weeks, followed by histologic assessment of bone formation. More specifically, we mix 1-5 μg of each FGF variant in 25 μl of water with 50 μl of 400 mg/ml fibrinogen, 24 μl of 5 U/ml thrombin, and 1 μl of 10,000 U/ml heparin in a 1 ml syringe. The fibrinogen / thrombin mixture carrier was developed by the American Red Cross and is marketed as ARC "Fibrin Sealant". Previous studies have shown that the fibrin sealant can be formulated to release trapped compounds, e.g. FGF-1, for up to 30 days. The fibrin / FGF-1 mixtures are incubated at room temperature for 2 hours to allow the fibrinogen to be cleaved by thrombin and form a firm gel. Mice are anesthetized with a mixture of xylazine and ketamine, the skin over the calvaria is opened and the preformed plug of fibrin sealant or fibrin sealant containing each FGF variant is placed over the calvaria. Subsequently, the skin is closed with wound clips. Two weeks later, the animals are sacrificed, the calvaria dissected and prepared for histology. Using this assay we compared the bone inductive affects of wild type FGF-1, Cys-free FGF-1, FGF-HBGAM chimera and Arg 136 » Lys-FGF. Figure 3 shows the amount of bone induced by each FGF variant. From these results we conclude that the Arg 136 » Lys mutant is the most osteoinductive followed by Cys-free > FGF-1 > FGF-HBGAM chimera. We presume that the Arg 136 » Lys mutant is more potent than the wild type FGF-1 due to its resistance to protease degradation.

A second series of experiments were designed to test the ability of FGF-1 to promote new bone formation directly in the intramedulary cavity. In this case, FGF-1 was mixed with fibrin sealant, as described above, except that the mixture was injected immediately, prior to the forming a gel, into the marrow space of anesthetized rat tibias. The injection needle entered the marrow directly through the skin and under the kneecap. We confirmed that the needle had entered the marrow cavity X-ray. Two weeks later the tibias of the rats were processed for histology. Figure 4 illustrates the presence of ectopic bone induced in the marrow as a response to FGF-1. This study illustrates the feasibility of administering FGF directly into bones that are at risk of fracturing.

Key Research Accomplishments

- Produced one new FGF-1 variant for a total of three FGF-1 variants since the beginning of this grant
- Established two new models for localized bone induction
- Established osteoblast migration and attachment assays (previous years)
- Established stem cell differentiation assays (previous years)
- Identified FGF-1 variants with enhanced anabolic activity for bone in osteoporosis model
- Established histological analysis of mineralized sections
- Demonstrated reduced hypotensive activity of cys-free mutant (previous years)

Reportable Outcomes

- Tissue Engineering/Regenerative Healing/Stem Cell Biology Conference
  Cambridge Healthtech Institute, Pittsburgh, Pennsylvania
  Title: Fibroblast Growth Factor-1: Multiple Aspects of Bone Formation.
- D.J. Mackenzie, R. Sipe, D. Buck, W. Burgess, J. Hollinger. "Recombinant Human Acid Fibroblast Growth Factor and Fibrin Carrier Regenerate Bone." *J. Orthop. Res.* Submitted (1999).

Conclusions

Together, the data summarized in the body of this report and presented in previous progress reports demonstrate that the original hypothesis and statement of work were of merit and realistic; although since taking responsibility of this grant in June, I have focused on localized

use of FGF-1. We have directly tested the bone anabolic effects of all currently available FGF-1 variants. The challenge for the final year of this application will be to identify the combinations of these modifications that result in the optimal growth factor for the rat osteoporosis model.

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Effect of Thrombin Treatment On Wild Type and R136K Mutant FGF-1

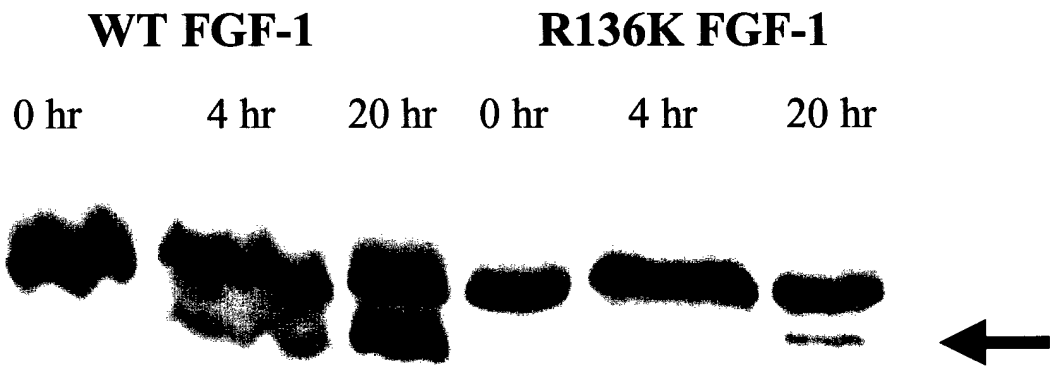


Fig. 1. The R136K mutation renders FGF-1 resistant to thrombin digestion. Twenty micrograms of wild type or R136K FGF-1 proteins were incubated at 37 °C with 6 U of thrombin in 600 µl reaction buffer (50 mM HEPES, 250 mM NaCl, 5 mM CaCl2). At indicated times, an aliquot was removed, boiled in sample buffer and ran on an SDS-PAGE follow by staining in coomassie blue. The lower band (arrow) represents the thrombin cleaved FGF. The relative intensity of the two bands in each lane, suggests that the R136K mutant is considerably more resistant to thrombin.

Mitogenic Activity of R136K FGF

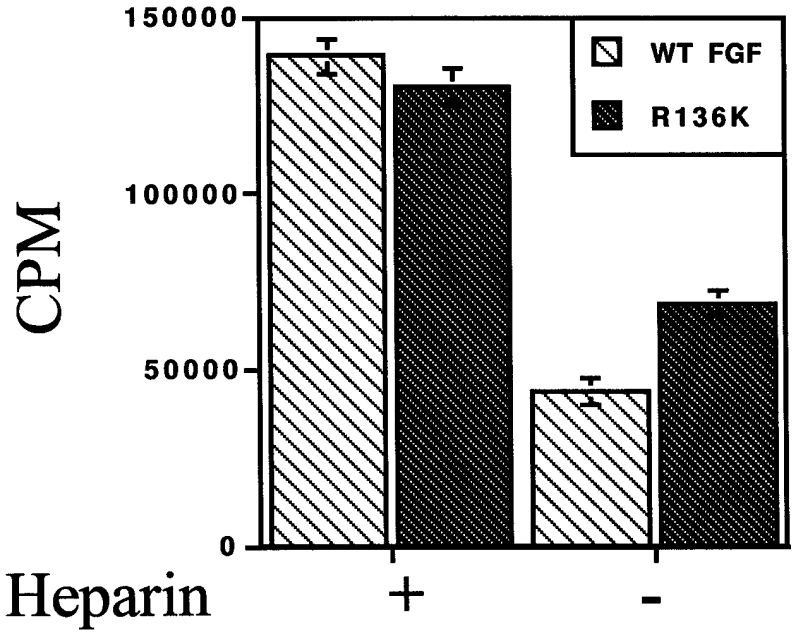


Fig. 2. The R136K mutant FGF-1 maitains its mitogenic activity. NIH3T3 cells were grown to 80% confluence, and transferred to medium containing 0.5% serum over night. Wild type FGF-1 or R136K mutant was added at 3 ng/ml without or with 10 U/ml heparin for 22 hrs. During the last 4 hrs of the incubation, 3H-thymidine was added at 0.5 μCi/ml. The cells were harvested and the amount of incorporated thymidine quantitated by scintilation counting. Results are from triplicates and error bars represent standard deviations.

Fibrin sealant carrier does not affect bone homeostasis

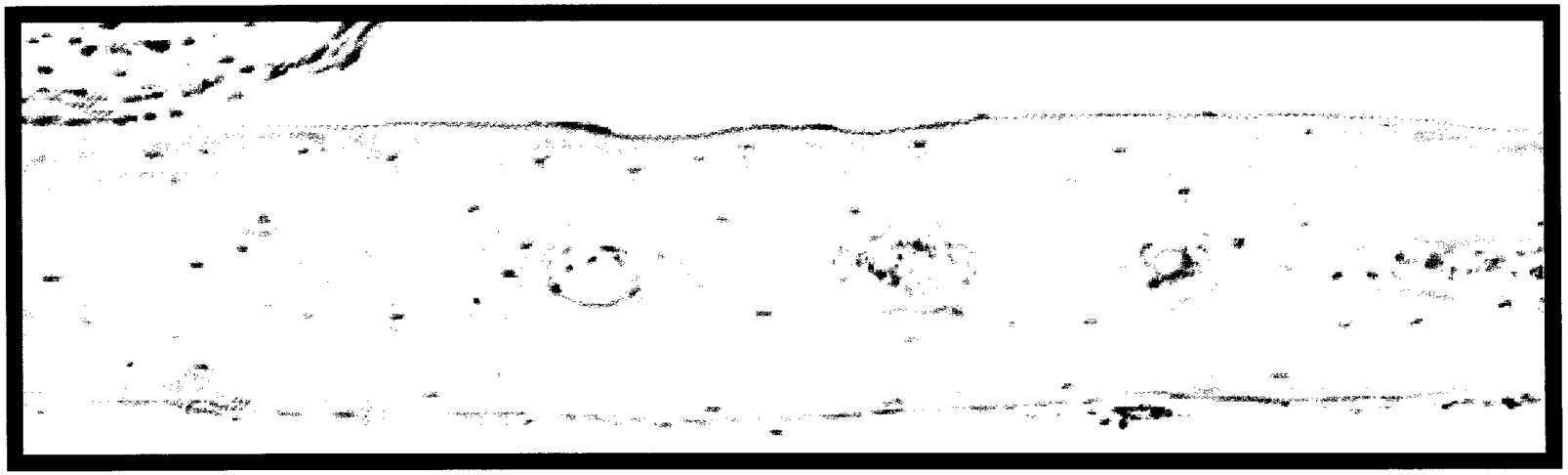
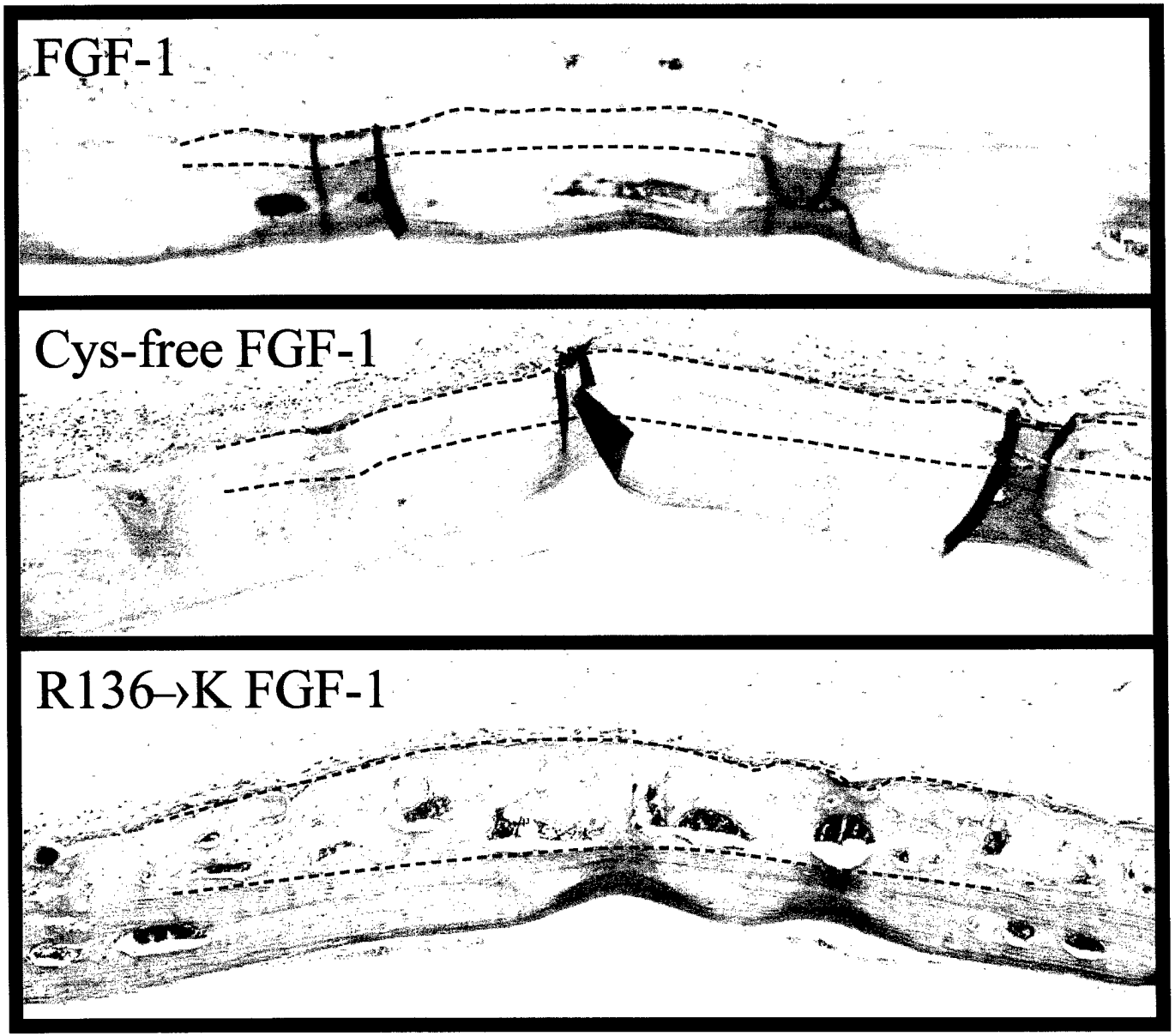


Fig.3a. Fibrin sealant carrier does not affect bone homeostasis. A plug of fibrin sealant consisting of 20 mg fibrinogen, 100 U thrombin, and 10 U heparin was placed on the calvaria of mice for 14 days. Subsequently the calvaria were dissected, fixed, decalcified, embedded in parafin, section and stained with H&E. This representative section illustrates that placement of fibrin sealant alone does not have any adverse affects on bone.

Bone induction by FGF-1 and its variants

Fig. 3b



Bone induction by FGF-1 and its variants

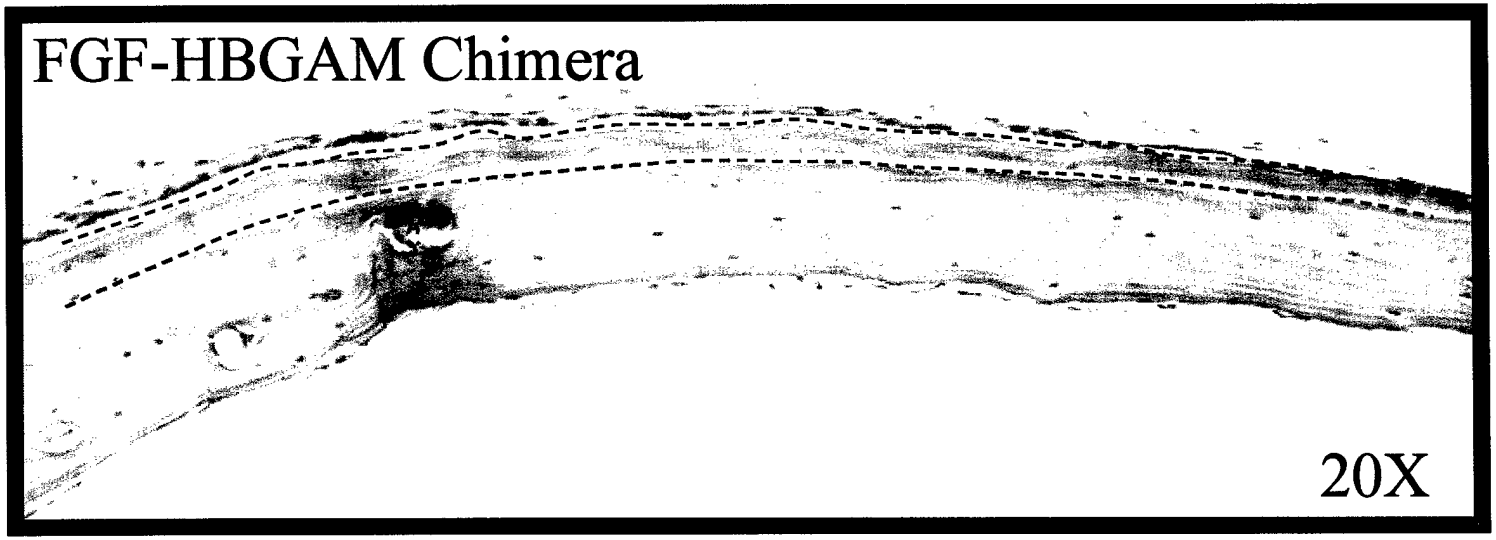


Fig. 3b. Bone induction by FGF-1 and its variants. A plug of fibrin sealant containing 5 μg of each FGF protein was placed on calvaria of mice as in Fig. 3a. Two weeks later the calvaria were examined histologically for evidence of new bone formation. In each panel the area of new bone is outlined by dotted lines.

FGF-1 induced bone formation in marrow space

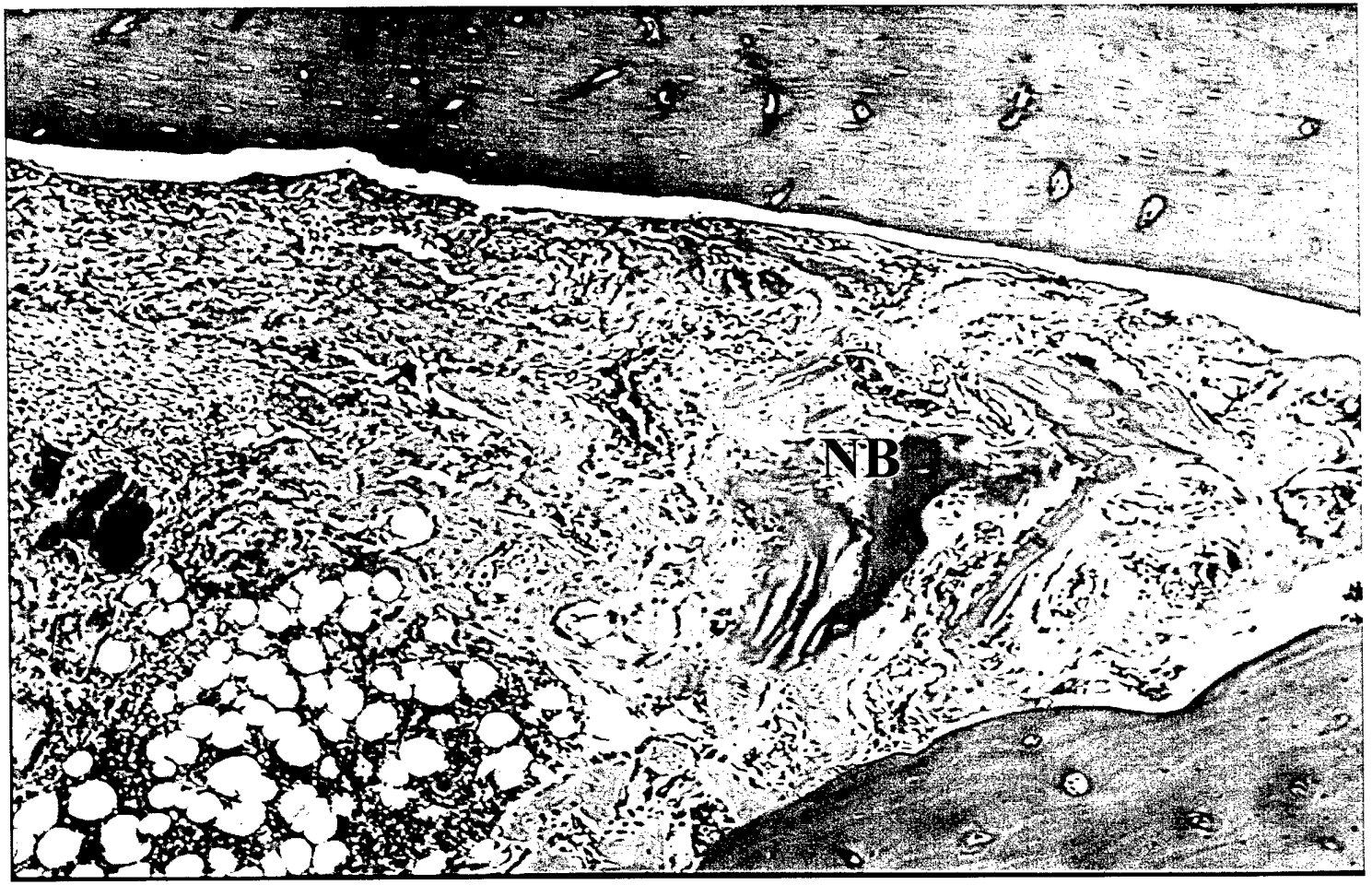


Fig. 4. FGF-1 was incorporated into fibrin sealant, and the mixture injected into the marrow space of rat tibias. Two weeks later, new bone formation was analyzed histologically. The area of newly formed bone is indicated by NB.